Exhibit E

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 42, Issue of October 19, pp. 39310–39319, 2001
Printed in U.S.A.
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Remodeling of the Major Pig Xenoantigen by *N*-Acetylglucosaminyltransferase III in Transgenic Pig*

Received for publication, May 14, 2001, and in revised form, July 31, 2001
Published, JBC Papers in Press, August 2, 2001, DOI 10.1074/jbc.M104359200

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We have been successful in generating several lines of transgenic mice and pigs that contain the human β-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase III (GnT-III) gene. The overexpression of the GnT-III gene in mice and pigs reduced their antigenicity to human natural antibodies, especially the Galα1–3Galβ1–4GlcNAc-R, as evidenced by immunohistochemical analysis. Endothelial cell studies from the GnT-III transgenic pigs also revealed a significant down-regulation in antigenicity, including Hanganutziu-Deicher antigen, and dramatic reductions in both the complement- and natural killer cell-mediated pig cell lyses. Changes in the enzymatic activities of other glycosyltransferases, such as α1,3-galactosyltransferase, GnT-IV, and GnT-V, did not support cross-talk between GnT-III and these enzymes in the transgenic animals. In addition, we demonstrated the effect of GnT-III in down-regulating the xenoantigen of pig heart grafts, using a pig to cynomolgus monkey transplantation model, suggesting that this approach may be useful in clinical xenotransplantation in the future.

The increasing problem of the worldwide shortage of donor organs has led to a revival of interest in xenotransplantation. The expression of complement regulatory proteins, such as membrane cofactor protein (CD46) (1), decay accelerating factor (CD55) (2), and CD59 (3, 4) in transgenic pigs, has been shown to be very effective in protecting against hyperacute rejection in a xenograft (5–8).

However, since Galili *et al.* reported that the Galα1–3Galβ1–4 GlcNAc-R (α-Gal)[1] is the major antigen to human xenotransplantation in pig, genetic approaches to modify this glycoantigen have been the focus of xenotransplantation studies. This antigen was first described as an internal type 1 chain but was later corrected as a linear type 2 oligosaccharide, the α-Gal that is synthesized by α1,3-galactosyltransferase (α1,3GT) (9–13). The human sequence, however, has suffered a deletion of a single nucleotide at two separate positions, which disrupts the translational reading frame (14, 15). As a result, humans produce a natural antibody that comprises as much as 1% of the circulating IgG and which is also found in significant amounts as an IgM antibody (16).

The most reliable approach for the elimination of α-Gal from pig tissue is to disrupt the pig α1,3GT gene via homologus recombination and/or gene transfer. However, gene targeting is not feasible at the present time. Another strategy for down-regulating the α-Gal involves taking advantage of enzymatic competition involving terminal glycosylation between α1,3GT and other glycosyltransferases for the common acceptor substrate in the *trans*-Golgi stack and network. Several glycosyltransferases, such as α1,2-fucosyltransferase (α1,2FT) (17, 18), α1,3-fucosyltransferase (α1,3FT), α2,3-sialyltransferase (α2,3ST) (19), and α2,6-sialyltransferase (α2,6ST) represent possibilities (20).

The strategy we present in this paper involves controlling sugar chain biosynthesis using the β-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase III (GnT-III) (21, 22), which leads to a remodeling of the total antigenicity of the cell surface (23). The mechanism by which the introduction of the GnT-III gene significantly suppresses xenoantigens is not fully understood, but its suppression could, in part, be caused by the inhibition of further branching of the carbohydrate moieties and/or a lack of maturation in processing; that is, once a bisecting GlcNAc residue is added to the core mannose by GnT-III, competitive enzymes, including α-3-D-mannoside β-1,4-N-acetylglucosaminyltransferase IV (GnT-IV) and α-6-D-mannoside β-1,6 N-acetylglucosaminyltransferase V (GnT-V), are prevented from introducing any further tri-structures

* This work was supported in part by grants-in-aid from the Ministry of Education, Science, and Culture of Japan and the Program for the Promotion of Basic Research Activities for Innovative Biosciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: α-Gal, Galα1–3Galβ1–4GlcNAc-R; α1,3GT, α1,3-galactosyltransferase; α1,2FT, α-1,2-fucosyltransferase; α1,3FT, α1,3-fucosyltransferase; α2,3ST, α2,3-sialyltransferase; α2,6ST, α2,6-sialyltransferase; GnT-III, β-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase III; GnT-IV, α-3-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase IV; GnT-V, α-6-D-mannoside β-1,6 *N*-acetylglucosaminyltransferase V; PEC, pig endothelial cell; PBS, phosphate-buffered saline; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high performance liquid chromatography; NHS, normal human serum; Ab(s), antibody(ies); mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; GS-IB4, *Griffonia simplicifolia* I; H-D, Hanganutziu-Deicher; FACS, fluorescence-activated cell sorter; LDH, lactate dehydrogenase; NK cell(s), natural killer cell(s); CH50, complement hemolytic activity; NeuNGc, *N*-glycolylneuraminic acid; CMP-NeuNAc, CMP-*N*-acetylneuraminic acid.

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in the Golgi stack (24). Our previous structural analysis of *N*-linked sugars of the pig endothelial cell (PEC) transfectant with GnT-III revealed that the complex type oligosaccharides with bi-, tri-, and tetraantennary structures, which contained α-Gal, decreased markedly with a parallel increase in bisected structures that contained no α-galactosyl residues (24, 25).

In the present study, transgenic mouse and pig lines carrying GnT-III were produced, and the expression levels of GnT-III, as well as changes in antigenicity in the various tissues, were analyzed.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—A cDNA of human GnT-III was subcloned into the pCX vector; a β-actin promoter and a cytomegalovirus enhancer (26). The plasmid was separately transformed into *Escherichia coli* C600 and then amplified using standard techniques.

Transgenic Mice and Pigs—B6C3F1 female mice were induced to superovulate and then crossed with B6C3F1 males. Microinjection and embryo transfer were performed by standard methods to generate transgenic mice. The DNA fragments for microinjection were prepared by digesting the plasmids with *Sal*I and *Hin*dIII to remove the vector sequences. DNA fragments were microinjected into mouse ova (C57BL/6 ×C3H), resulting in transgenic mice. Genomic DNA from the tail tips of newborn mice was analyzed by polymerase chain reaction and Southern blots to identify the produced transgenic animals. Mice carrying these pCX-GnT-III plasmids were crossed with B6 to obtain offspring.

The pCX-GnT-III gene was also used to produce transgenic pigs. Prepubertal cross-bred gilts (Large White/LandraceXDuroc) were used as embryo donors and recipients. Methods used in the superovulation for gilts have been presented previously (27). Embryo donors were artificially inseminated, and embryos were collected 50–54 h after human chorionic gonadotropin injection. Embryos were centrifuged at 12,000 × *g* for 8 min to visualize the pronuclei and microinjected with several thousand copies of the hybrid gene. Microinjected embryos were then transferred to unmated synchronized recipients or the embryo donors (donor-recipients). Transgenic pigs were identified by polymerase chain reaction and/or Southern blot analysis with genomic DNA extracted from the tail tips of the newborn pigs. Founder transgenic pigs were bred with nontransgenic boars or gilts to obtain the second generation.

Glycosyltransferase Assays—For the assay of enzyme activity, tissues were sonicated and lysed in PBS. The enzyme activities of GnT-III, GnT-IV (28), and GnT-V (29) were determined using the pyridylaminated biantennary sugar chain GlcNAcβ1–2Manα1–6 (GlcNAcβ1–2Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc-PA as a substrate (30, 31).

The reaction buffer for the GnT-III assay consisted of 125 mM MES buffer, pH 6.25, containing 40 mM UDP-GlcNAc, 20 mM $MnCl_2$, 400 mM GlcNAc, and 1% Triton X-100. The reaction mixture for GnT-IV contained 250 mM MOPS buffer, pH 7.3, 80 mM UDP-GlcNAc, 15 mM $MnCl_2$, 400 mM GlcNAc, and 1.0% (W/V) Triton X-100. Assayed GnT-V activity employed, pH 6.25, 250 mM MES buffer, containing 80 mM UDP-GlcNAc, 20 mM EDTA, 400 mM GlcNAc, and 1.0% (W/V) Triton X-100. It should be noted that Mn^{2+} is not essential for GnT-V activity. 20 mM EDTA, contained in the reaction mixture, completely inhibited GnT-III activity. To 25 μl of these solutions 10 μl of 100 μM substrate was added followed by 15 μl of cell lysate. The assay mixture was then incubated at 37 °C for 3 h (28).

The acceptor substrate, pyridylaminated lacto-*N*-neotetraose (Galβ1–4GlcNAcβ1–3Galβ1–4Glc-PA) at a final concentration of 10 μM was employed in the α1,3GT activity assays. Lacto-*N*-neotetraose was purchased from Seikagaku Kogyo (Tokyo, Japan) and pyridylaminated according to the method of Kondo *et al.* (32). α1,3GT activity was assayed in a reaction mixture containing 10 μM HEPES, pH 7.2, 20 mM UDP-galactose, 10 mM $MnCl_2$, 33 mM NaCl, and 3 mM KCl. 10 μl of 50 μM substrate and 15 μl of cell lysate were added to this mixture, which was then incubated at 37 °C for 3 h (19).

The enzyme reactions were quenched by boiling for 5 min. The samples were then centrifuged at 12,000 × *g* for 5 min, and an aliquot of each supernatant was subjected to HPLC analysis, using a TSK-gel ODS-80TM column (4.6 × 250 mm). The reaction products were eluted with 20 mM acetate buffer, pH 4.0, containing *n*-butyl alcohol at a flow rate of 1.0 ml/min at 55 °C and were monitored with a fluorescence spectrophotometer (Shimadzu model RF-10AXL, Tokyo) using excitation and emission wavelengths of 320 and 400 nm, respectively. The specific activity of the enzyme is expressed as mol of product produced per h of incubation per mg of protein. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL), using bovine serum albumin as a standard.

Immunohistochemical Detection of α-Gal—Various organs were excised from transgenic mice and pigs. A portion of each organ was fixed with 4% paraformaldehyde and Dulbecco's PBS for 30 min. The fixed sections were incubated with blocking solution (2% bovine serum albumin and Dulbecco's PBS) for 1 h and then reacted with normal human pooled serum (NHS) of blood type O or a mouse mAb anti-α-Gal, M86 (a generous gift from Dr. U. Galili) (33). After removal of excess antibody, the sections were reacted with FITC-conjugated goat anti-human Ig (Cappel, West Chester, PA), or FITC-conjugated rabbit anti-mouse IgM (Cappel ICN, Aurora, OH), respectively. Each section was also reacted FITC-conjugated *Griffonia simplicifolia* I (GS-IB4) lectin, which binds the α-Gal (Honen, Tokyo). Double staining of pancreas islets was also carried out using anti-GnT-III mAb (Fujirevio, Tokyo) and anti-pig insulin polyclonal Ab (DAKO Japan, Kyoto), and subsequently stained with FITC-conjugated anti-mouse Ig (Cappel ICN) and Alexa Fluor 594-labeled goat anti-guinea pig IgG secondary antibody (Molecular Probes Europe BV, Leiden, The Netherlands), respectively. For the detection of monkey C3 and C5b-9 deposition, mouse anti-human C3 mAb and mouse anti-human C5b-9 mAb (DAKO Japan) were used as the first antibody and subsequently stained with FITC-conjugated rabbit anti-mouse IgG (Cappel ICN). The slides were viewed by means of a Zeiss Axioplan 2 universal microscope (Jena, Germany).

Flow Cytometry—The PEC from transgenic pigs with or without human GnT-III was removed from the aorta and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with L-glutamine (Life Technologies, Inc., Rockville, MD) and penicillin/streptomycin (Meiji, Tokyo) (34). The PECs were incubated with various dilutions of NHS at 4 °C for 1 h, washed, and then incubated with 1.25 μg of FITC-conjugated anti-human Ig (Cappel) as a second antibody for 1 h at 4 °C. The cell surface carbohydrate epitopes were also examined with an FITC-conjugated GS-IB4 lectin (Honen) and chicken anti-Hanganutziu-Deicher (H-D) antigen polyclonal Ab (a gift from Dr. N. Wakamiya, Osaka University, Osaka, Japan) and FITC-conjugated rabbit anti-chicken IgG (Cappel). The stained cells were analyzed with a FACS Calibur flow cytometer (Nippon Becton Dickinson, Tokyo).

Lactate Dehydrogenase (LDH) Assay—This assay was performed according to the manufacture's recommended protocol, using an MTX LDH kit (Kyokuto, Tokyo). The PEC from transgenic pigs was plated at 2×10^4 cells/well in flat bottomed gelatin-coated 96-well trays 1 day prior to assay. Fifteen hours after plating the cells, the wells were washed twice with serum-free Dulbecco's modified Eagle's medium to remove the LDH, which is present in fetal calf serum, and incubated with several concentrations of NHS that had been diluted with Dulbecco's modified Eagle's medium. The plates were incubated for 2 h at 37 °C and the released LDH was then measured. The percent cytotoxicity was calculated using Equation 1

$$\text{Cytotoxicity} = (E - N - S)/(M - N - S) \times 100 \qquad \text{(Eq. 1)}$$

where E is the experimentally observed release of LDH activity from the target PEC, N the LDH activity in each concentration of NHS, S the spontaneous release of LDH activity from target PEC incubated in the absence of NHS, and M the maximal release of LDH activity, as determined by sonication.

The spontaneous release of LDH activity from PEC was less than 5%, compared with the maximal release obtained by sonication (34).

NK Cell-mediated Cytotoxicity Assay—The PEC from transgenic pigs were plated at 2×10^4 cells/well in a flat bottomed gelatin-coated 96-well plate. Fifteen hours after plating the cells, the plates were incubated with effector cells, an NK-like cell line, YT cells, which were kindly provided by Drs. Junji Yodoi and Keisuke Teshigawara (University of Kyoto) (35), at various effector:target ratios. Each assay was performed in triplicate. After a 4-h incubation at 37 °C, the released LDH was measured using an MTX LDH kit (Kyokuto). The spontaneous release of LDH activity from effector cells and target cells were less than 10 and 5%, respectively. The results are expressed as the percent of specific lyses (36).

Experimental Animals—Examinations were carried out for according to the guidelines for the handling of animals from the Research Institute of the HAMRI Co., Ltd. (Ibaraki, Japan). The heterozygous transgenic pigs with GnT-III and wild-type controls, either sex (18–22 days old, 2.5–6.0 kg), were used as donors for all experiments. Cynomolgus monkeys, *Macaca fascicularis*, obtained from HAMRI Co., Ltd., weighing 2.5–7.0 kg, were used as recipients. Preoperative serum from all monkeys was assayed for the anti-pig endothelial cell antibody titer and

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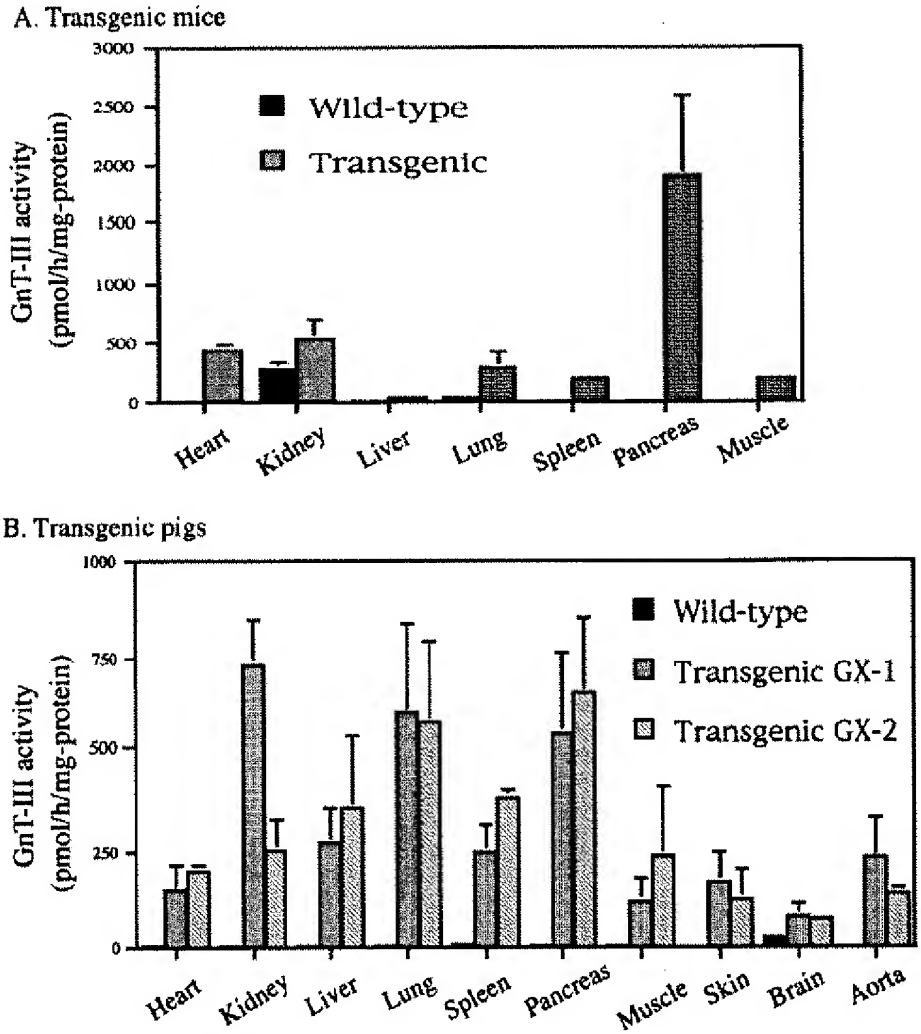

FIG. 1. **Features of GnT-III enzyme activity in transgenic mice and pigs.** GnT-III activity in each organ of wild-type and transgenic animals, mice (*panel A*) and pigs (*panel B*), is shown. GnT-III activity (pmol/h/mg of protein) was examined. Values are the mean ± S.E. of triplicate determination.

complement hemolytic activity (CH50 unit) (37).

Anti-PEC Natural Antibodies and CH50 in Cynomolgus Monkey—The anti-PEC antibodies, IgG and IgM, were checked, using PEC as a target. The PECs from control pigs were incubated with 10% serum from each recipient monkey at 4 °C for 1 h, washed, and then incubated with 1.25 μg of FITC-conjugated anti-human IgG or IgM (Cappel) as a second antibody for 1 h at 4 °C. Stained cells were analyzed with a FACS Calibure flow cytometer.

CH50 was determined by a microtiter method, according to the methodology described by Mayer (37, 38). In this procedure, CH50 was assayed in gelatin Veronal buffer by using sensitized sheep erythrocytes. After incubation at 37 °C for 60 min, 50 μl of the same buffer was added, and the mixtures were centrifuged. The hemoglobin content of each supernatant was estimated spectrophotometerically. The CH50 unit was defined as the serum volume sufficient to lyse 50% of the erythrocytes added to each well, and the complement activity in a test serum was then calculated as the number of CH50 units.

Heterotopic Heart Transplantation—After the donor pigs had been anesthetized with thiopental sodium (20 mg/kg) and Stresnil (2 mg/kg), a median sternotomy was performed. The inferior vena cava was divided above the diaphragm, and 200 ml of glucose-potassium cardioplegic solution with heparin (1,000 IU) (39) was then infused from the ascending aorta. The heart was excised under topical cooling with PBS (4 °C) after division of the superior vena cava, the pulmonary aorta and veins, and the ascending aorta.

Recipient cynomolgus monkeys were anesthetized using ketamine hydrochloride (10 mg/kg) and xylazin (1 mg/kg). A heterotopic heart transplantation was performed in the abdomen, according to the Ono-Lindsey method (40). A midline abdominal incision was used to expose the aorta and inferior vena cava below the renal vessels. The donor heart was placed in the abdomen of the recipient monkey, the end-to-side anastomosis being donor aorta to recipient abdominal aorta and donor pulmonary aorta to recipient inferior vena cava. No immunosuppression nor anticoagulants was administered before or after the procedure.

The rejection of the cardiac transplants was defined based on the cessation of beating. The following time was 4 h after the operation. Samples of graft tissue were removed at the time of rejection or 4 h after transplantation for immunohistochemical analysis.

Statistics—Data are presented as the mean ± S.E. Student's *t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Establishment of Transgenic Mice and Pigs—The pCX promoter was used for the ubiquitous expression of GnT-III in transgenic mouse. Eight founder pCX-GnT-III transgenic mice were obtained from 73 live pups from 576 microinjected oocytes. A founder that was determined to express high levels of human GnT-III was mated with B6 mice to propagate transgenic offspring for the analysis of transgene expression in various tissues.

Transgenic pigs were also obtained by means of the pCX-GnT-III construct. Five founders of pCX-GnT-III transgenic

TABLE I
Immunohistochemical analysis
Grading scale: −, not stained; ±, stained equivocally or weakly; +, stained moderately; ++, stained intensely.

Animal	NHS		GS-IB4		M86	
	Wild-type	Transgenic	Wild-type	Transgenic	Wild-type	Transgenic
Transgenic mice						
Heart						
1	++	+~±	++	±	++	+
2	++	+	++	+~±	++	+
Kidney						
1	+	+	+	+	+	+
2	+	+	+	±	+	+
Liver						
1	+	±~−	+	±~−	+	±~−
2	++	±	++	−	+	±~−
Lung						
1	+	−	+	±~−	+	−
2	+	−	±	−	±	±~−
Pancreas						
1	±~−	−	±	−	±~−	−
2	+~±	−	+	−	+	−
Muscle						
1	+~±	−	+	±	+	±~−
2	++	+~±	++	±	++	+~±
Transgenic pigs						
Heart						
1	++	±	+	±	+	−
2	+	±	+	±	+	±
3	++	±	+	±	+	−
Kidney						
1	++~+	±	+	±	++~+	±
2	+	±	++	+~±	+~±	−
3	++	±~−	++	±~−	+	−
Liver						
1	++	+	+	±	++	±
2	++	±	+	±~−	++	+~±
3	++	+	++	+	+	±
Lung						
1	+	±	±	±	+	±~−
2	+	−	+	±	+	±
3	+	±	+~±	±	+	±~−
Pancreas						
1	+	−	+	±	+	−
2	ND[a]	ND	ND	ND	ND	ND
3	±	−	±	−	−	−
Spleen						
1	+	±	+	±	±	−
2	+	+	+	−	+	±
3	+	±	+	±	+	±
Muscle						
1	+	±	±	±	++~+	±
2	±	−	+	−	+~±	±
3	++~+	+	+	±	+	±~−
Skin						
1	±	−	−	−	±	±
2	−	−	±	−	−	−
3	+	±	−	−	±	−
Brain						
1	−	−	−	−	−	−
2	−	−	±	−	−	−
3	±	−	−	−	−	−
Aorta						
1	+	+	++	+	+	±
2	ND	ND	++	+	++~+	±
3	++	+	++	±	++	++~+

[a] ND, not determined.

pigs were obtained from 59 live pups from 583 microinjected oocytes. Of the founder pigs, one was stillborn, and another died shortly after birth. Two founders, Gx-1 and Gx-2, showed GnT-III enzyme activity in their tails, and the other, Gx-3, had only a very low level of GnT-III activity. The transgenic pig, Gx-1, was successfully bred, and three offspring, 3 weeks (pig 1), 3 months (pig 2), and 6 months (pig 3), with the transgene were examined for *in vitro* study. Two, 3 weeks, were used in the transplantation experiments.

Copy Number of Transgenes—The copy number of transgenes in hemizygous offsprings (F1) of transgenic mouse and pig lines was examined by Southern hybridization. The copy number of transgenic mouse, line 2, which was used in this study, had seven copies of pCX-GnT-III constructs, and the pig lines GX-1 and GX-2 had three and two copies, respectively. The level of activity of GnT-III in each animal was not correlated with the copy numbers of the transgene.

Profiles of the GnT-III Transgenic Mice and Pigs—The profiles of the GnT-III activities of each organ in wild-type and the GnT-III transgenic animals were investigated. Although the

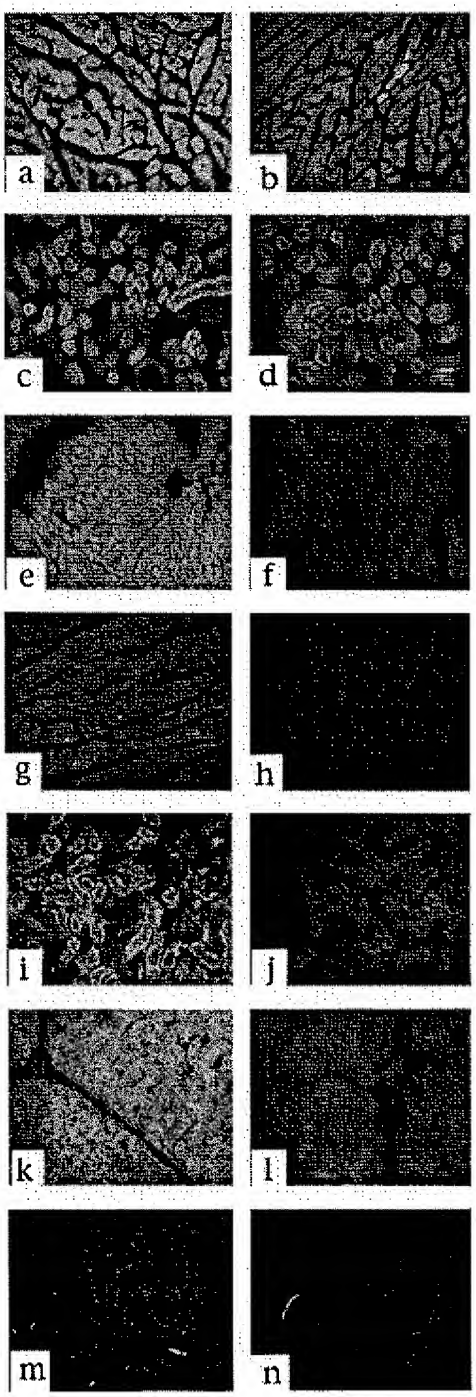

FIG. 2. **Immunostaining of tissue sections from control and transgenic animals, mice and pigs.** Staining with GS-IB4 lectin (*panels a, b, e, f, m,* and *n*), M86 (*panels c, d, g,* and *h*), and NHS (*panels i, j, k,* and *l*) is shown on tissue sections from heart (*panels a, b, g,* and *h*), kidney (*panels c, d, i,* and *j*), liver (*panels e, f, k,* and *l*), and brain (*panels m* and *n*), from the wild-type mouse (*panels a, c,* and *e*), the GnT-III transgenic mouse (*panels b, d,* and *f*), the wild-type pig (*panels g, i, k,* and *m*), and the transgenic pig (*panels h, j, l, n*). Representative fields from each section were examined.

wild-type mouse showed GnT-III activity only in kidney tissue, GnT-III was expressed ubiquitously in the GnT-III transgenic mice (Fig. 1*A*).

As shown in Fig. 1*B*, the wild-type pigs showed very low levels of GnT-III activity in the kidney, but a slightly higher level in the brain. The CAG promoter led to the nearly ubiquitous expression of GnT-III in the organs of the transgenic pig.

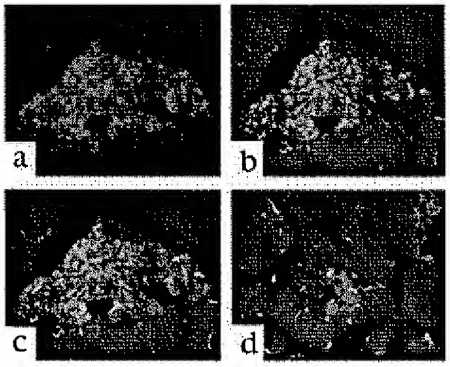

FIG. 3. **Double staining with anti-pig insulin Ab and anti-GnT-III.** Staining with anti-pig insulin (*panels a, c,* and *d*) and anti-GnT-III (*panels b, c,* and *d*) is shown on pancreas tissue sections from control (*panel d*) and transgenic pigs (*panels a, b,* and *c*). Representative fields from each section were examined.

Immunohistochemical Study—To analyze the alteration of antigenicity in the transgenic mouse, immunostaining of each organ was performed using NHS and GS-IB4 lectin and M86 monoclonal antibody (Table I). Characteristic of the transgenic mouse, changes of antigenicity were found in most organs except for the kidney, which has endogenous GnT-III activity (Fig. 2, *c* and *d*). In particular, the antigenicity of the liver from the GnT-III transgenic mouse is clearly down-regulated despite its relatively lower expression of GnT-III activity (Fig. 2, *e* and *f*).

In the case of pigs, compared with wild-type pig, GnT-III transgenic pigs indicated a lower susceptibility to NHS, GS-IB4 lectin, and M86 mAb in many organs except brain, which also had GnT-III endogenous enzyme activity (Fig. 2, *m* and *n*).

Double Staining of Pancreas Tissue—To determine whether the pancreatic islets from the transgenic pig have elevated GnT-III activity, double staining with anti-GnT-III Ab and anti-insulin Ab was carried out. Double staining of the pancreas revealed that the islets from the transgenic pig have a high level of expression of the GnT-III enzyme (Fig. 3).

Cross-talk of the Enzymes—The α1,3GT and GnT-IV, and GnT-V enzyme activity in the control animals and the influences of an excess of GnT-III over the enzymes in each organ were measured.

The average GnT-IV activities in many organs of the transgenic mouse and pig were lower than those in wild, but the differences were not significant (Fig. 4, *A* and *D*). It was not possible to predict changes in GnT-V and α1,3GT activities in both mice and pigs.

Profiles of PEC from the Transgenic Pigs—GnT-III and α1,3GT activities in the PEC from transgenic piglets were examined by HPLC, and the amelioration of antigenicity of the PEC was also analyzed by flow cytometry, using NHS, GS-IB4 lectin, and M86 (Fig. 5, *A–E*).

Although control parental PEC reacted strongly with human natural antibodies in NHS, the PEC from GnT-III transgenic pigs showed a diminished reactivity. Consistent with the previous *in vitro* study, the PEC from *in vivo* also ascertained the clear down-regulation of antigenicity. The percent reduction in xenoantigenicity to human antibodies was ~50%, as evidenced by mean fluorescence intensity. The α-Gal was ~50% by GS-IB4 and to 70% by M86 down-regulated in PEC from the GnT-III transgenic pigs.

H-D Antigen—The remodeling of pig antigenicity by overexpression of GnT-III is directed not only at the α-Gal but also at other unknown epitopes. The influence on the H-D antigen in the PEC isolated from GnT-III transgenic pig was then examined. As expected from our previous study, the H-D antigen on

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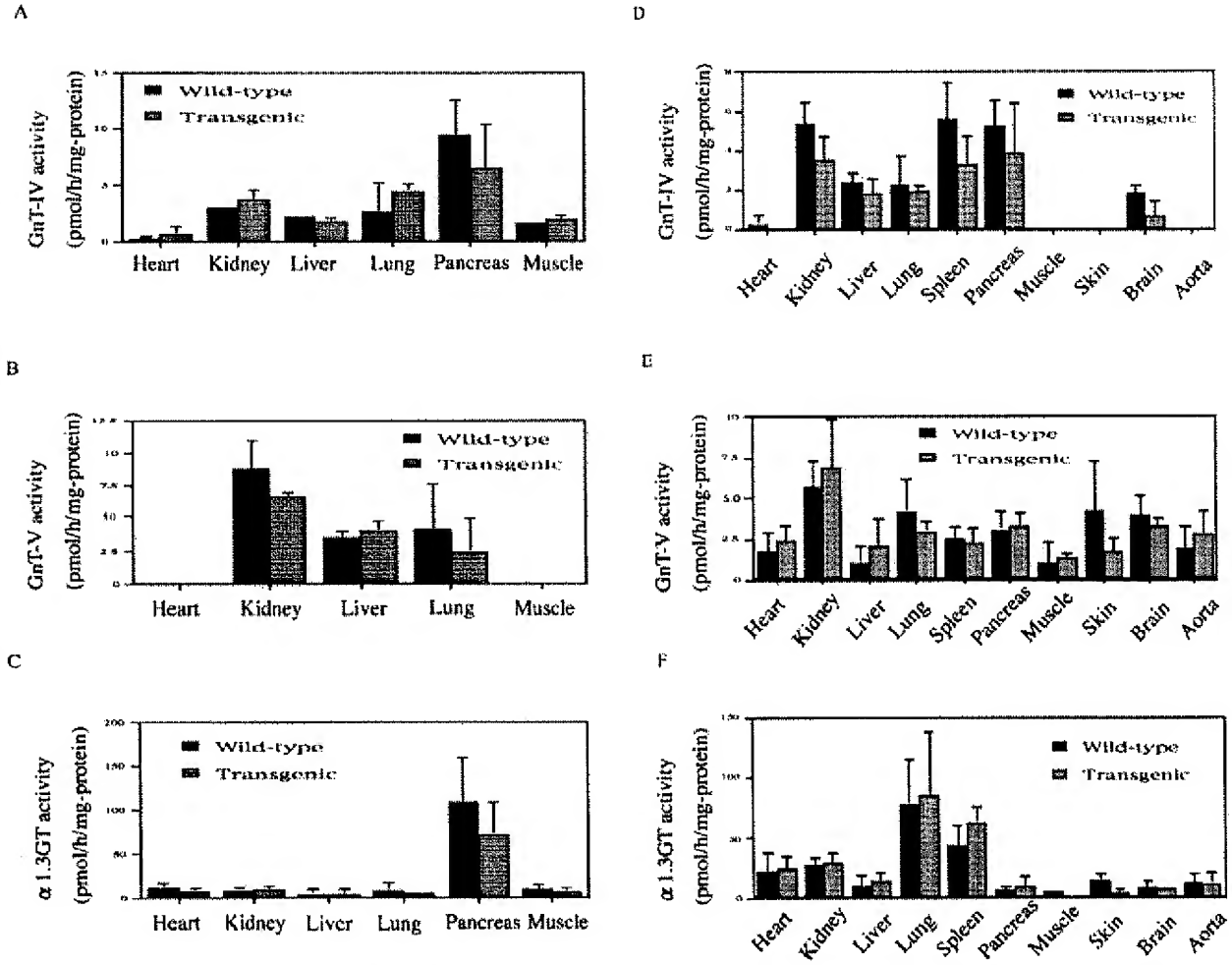

FIG. 4. **Changes in several enzyme activities in GnT-III transgenic animal tissues, mice tissues (*panels A, B,* and *C*) and pig tissues (*panels D, E,* and *F*).** To assess the influence of the GnT-III transgene on intrinsic GnT-IV, GnT-V, and α1,3GT activities, relevant enzyme activities were measured. Compared with the enzyme activity in wild-type organs, those in transgenic animals indicated small and variable changes.

PEC was significantly down-regulated (Fig. 5*F*).

Complement-mediated and NK-mediated Cytotoxicities of the PEC—The amelioration of complement-mediated lysis as a result of the overexpression of GnT-III was determined. In these experiments, NHS was used as a source of natural antibody and complement, as an *in vitro* model of hyperacute rejection. An approximate 40% inhibition of cytotoxicity was observed in the PEC derived from the GnT-III transgenic pig, GX-1 (Fig. 5*G*). These results also suggest that GnT-III is quite effective in the remodeling of PEC.

To examine the involvement of the remodeling of oligosaccharides in cell-mediated cytotoxicity, assays for NK cell-mediated direct cytotoxicity were also carried out using the PEC from the GnT-III transgenic pigs as a target. As expected, cytotoxicity to the PEC isolated from GnT-III transgenic pig was decreased substantially compared with that from the wild-type pig (Fig. 5*H*).

Experimental Xenotransplantation of Pig to Cynomolgus Monkey—Experimental xenotransplantation of pig to cynomolgus monkey was performed using the heart from the GnT-III transgenic pigs. The natural antibody titer of each cynomolgus monkey was evaluated using the PEC, which is derived from the wild-type pig. The CH50 unit was also measured, and these data are summarized in Table II.

An abdominal heterotopic heart transplant was achieved. The ischemic time of the transplant organ varied from 35 to 60 min. Two hearts from wild-type pigs, which were transplanted to cynomolgus monkeys, were hyperacutely rejected.

On the other hand, one heart graft carrying human GnT-III transplanted into the monkey with high natural Ab, especially IgM, was also rejected hyperacutely, whereas another heart graft from a transgenic pig continued to beat normally when removed at 4 h after transplantation for purposes of histological analysis.

In addition, immunostaining of C3 and C5b-9 deposition on the graft after rejection or 4 h after transplantation was performed. Compared with the rejected grafts, the specimen from the transgenic pig 2 organ showed a lower deposition of C3 as well as C5b-9 (Fig. 6).

DISCUSSION

The pCX promoter expressed human GnT-III ubiquitously and intensely in the vascular endothelia of transgenic pigs. With this construct, the frequency of live births was slightly decreased, suggesting the possibility of a slight deleterious effect of human GnT-III expressed under the regulation of this promoter on the early development of pigs. We were able to produce transgenic mice carrying the same constructs used in the present study with nearly equal frequency.

Immunohistochemical profiles revealed that this strategy,

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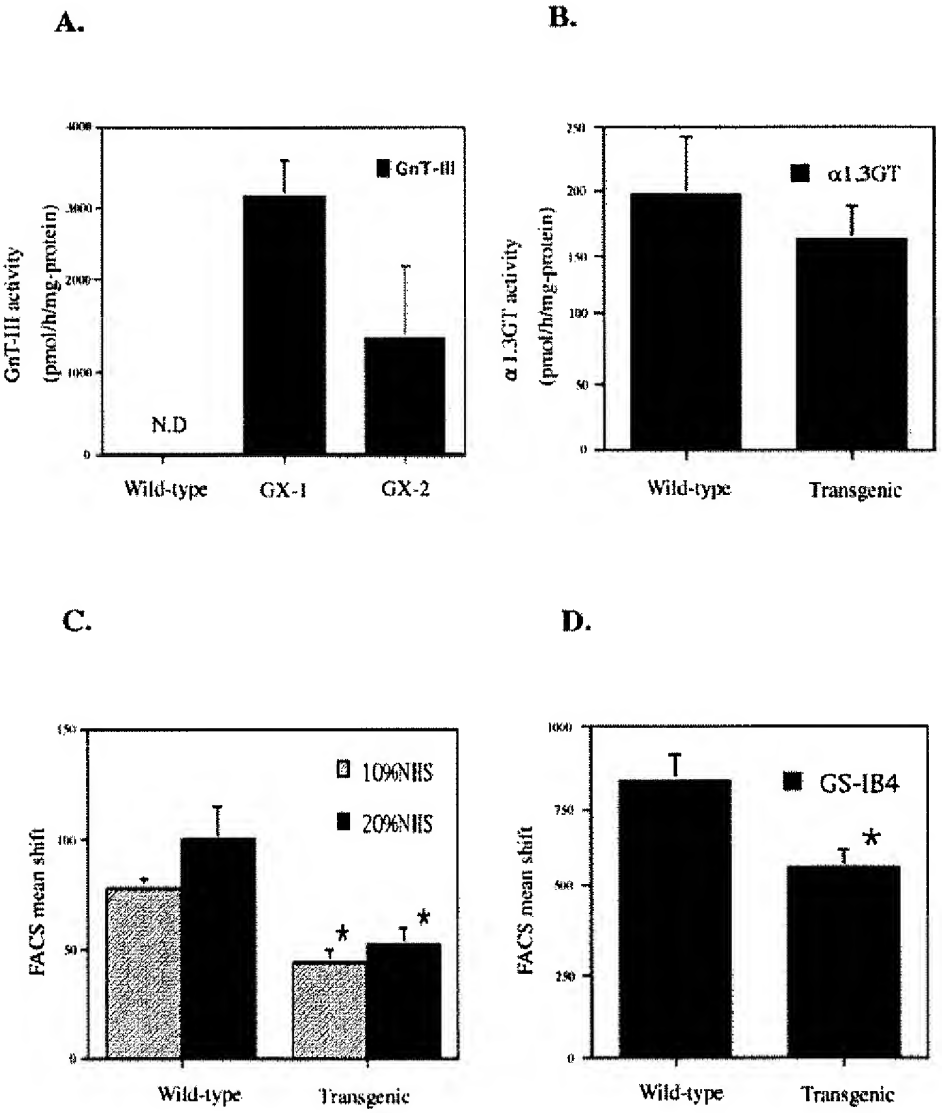

FIG. 5. **Features of the PEC from transgenic pigs.** Enzyme activities, GnT-III (*panel A*) and α1,3GT (*panel B*) of PECs from transgenic pigs were measured by HPLC. Each value is expressed as the mean ± S.E. of three to four independent experiments. Xenoantigenicity of transgenics to NHS (*panel C*), GS-IB4 (*panel D*), and M86 (*panel E*) were investigated by flow cytometry. PECs from control and transgenic pigs were treated with 10 or 20% NHS as the first antibody and anti-human immunoglobulin second antibodies. The reduction of α-Gal on the cell surface was also analyzed using GS-IB4 lectin and M86 mAb. PECs from control and transgenic pigs were treated with FITC-conjugated GS-IB4 lectin, M86 mAb, and FITC-conjugated anti-mouse IgM secondary antibody. The FACS mean shift value of the PEC treated with polyclonal chicken anti-H-D antigen antibody is indicated (*panel F*). The H-D antigen of the PEC from transgenic pigs was down-regulated significantly. Each value is expressed as the mean ± S.E. of six to eight independent experiments. The amelioration of complement-mediated lysis by from wild-type and transgenic pigs was estimated by 20 or 40% NHS, which served as a source of natural antibodies and complement (*panel G*). The percentage of inhibition of NK cell-mediated lysis is also presented. YT cells were incubated with PEC at effector:target ratios of 10:1 or 5:1 (*panel H*). The resulting cytolysis is expressed as the mean percent of specific lysis ± S.E. of six to eight independent experiments. * indicates significance ($p < 0.05$).

i.e. remodeling by GnT-III, is feasible in transgenic animals. Although each organ in the wild-type animals reacted strongly with human natural antibodies in NHS and GS-IB4, most organs in the transgenic pig showed a diminished reactivity because surface xenoantigens, especially α-Gal, were reduced. It is particularly noteworthy that the endogenous mouse GnT-III activity avoids remodeling the antigenicity in the transgenic mouse kidney despite the highly elevated level of expression of human GnT-III activity. On the contrary, the liver, with a relatively low elevated human GnT-III activity in the transgenic mouse, clearly showed a down-regulation in antigenicity.

On the other hand, the wild-type pigs, in which low endogenous GnT-III activity was observed in the kidney, led to an apparent down-regulation of antigenicity in the kidney of transgenic pigs. In the case of pig brain, the level of GnT-III is not distinct but is higher than that of other tissues. Therefore, similar to the mouse kidney, endogenous GnT-III activity might have hindered the remodeling of glycoantigen in this organ. Fortunately, the wild-type pig brain itself did not indicate a high level of antigenicity to human serum in the immunostaining procedure. These data suggest that transgenic pigs with GnT-III are suitable for kidney xenotransplanation and also have a possibility of therapeutic use for Parkinson's disease using the brain.

To address the issue of whether pancreatic islets from the GnT-III transgenic pig are able to be used for pancreatic islet transplantation, double staining was carried using anti-pig insulin and anti-GnT-III Abs. Fortunately, in addition to the

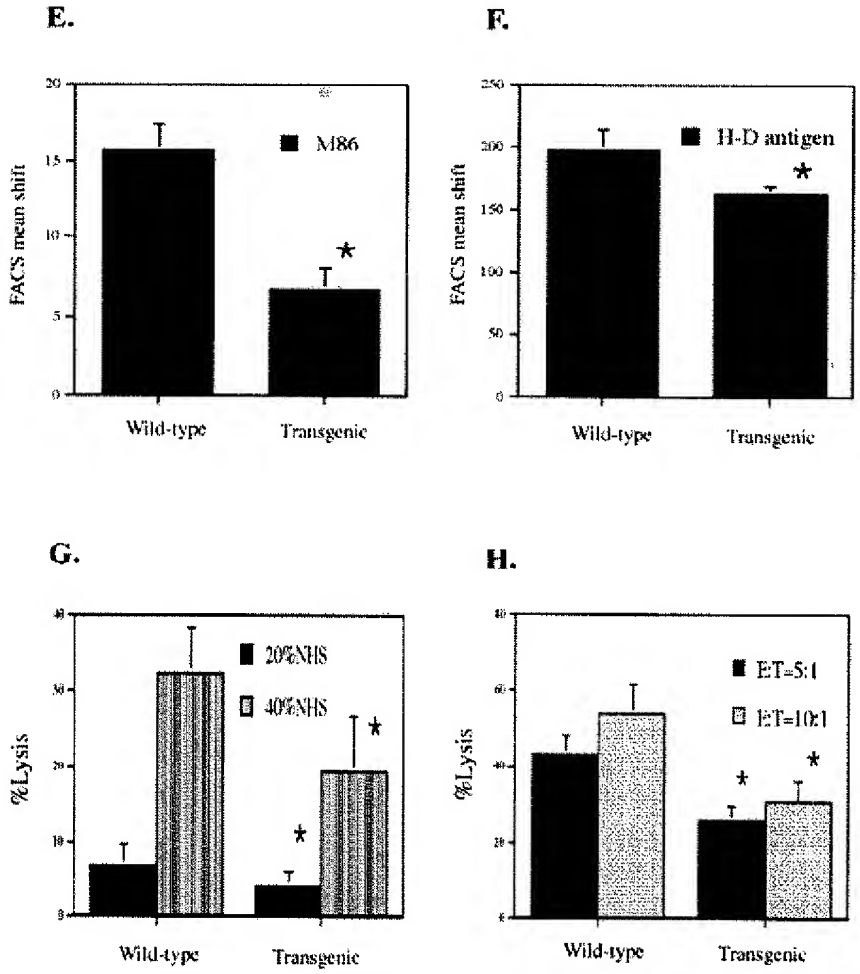

FIG. 5—*continued*

high level of GnT-III activity in entire pancreas tissue of the transgenic pig, the pancreatic islets clearly expressed GnT-III.

In our previous study, using the PEC transfectants with GnT-III, several clones with a high level expression of GnT-III indicated that the enzyme activity of α1,3GT, GnT-IV, and GnT-V is diminished (19). The possibility of enzymatic cross-talk between GnT-III and these enzymes was considered. However, *in vivo* results of these enzymes between control and transgenic animals failed to support this *in vitro* hypothesis. Concerning GnT-IV and α1,3GT, the average enzyme activities of most tissues, including PEC from transgenic animals, were lower than those from the wild-type. However, no significant differences were found in this study.

In terms of α1,3GT activity in organs, the mouse expresses a relatively lower α1,3GT activity than the pig in many organs, but the differences are in the 10-fold range, except for the lung. On the contrary, some mouse tissues, such as the pancreas, revealed a higher α1,3GT activity than that in pigs. The data may not support the reports by Galili and co-workers (41), who concluded that α-Gal epitope expression in pig organs is up to 500-fold higher than in mouse organs. However, the amount of α-Gal epitopes in each tissue may not be directly related to α1,3GT enzyme activity.

We also demonstrated the remodeling of antigenicity to human serum in PEC derived from the transgenic pigs, which is in agreement with conclusions reached in our previous *in vitro* study. The PEC from transgenic pig showed approximately a 50% reduction in the antigenicity by GnT-III, not only α-Gal

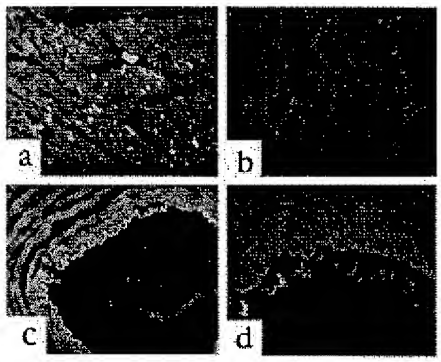

FIG. 6. **Deposition of activated complement components in pig heart xenograft.** Staining with monkey C3 (*panels a* and *b*) and C5b-9 (*panels c* and *d*) is shown for the pig tissue section from the controls 2 (*panel a*) and 1 (*panel c*) and GnT-III transgenic 2 (*panels b* and *d*). Representative fields of coronary and ventricle from each section were examined. C3 and C5b-9 are both reduced on the specimen for transgenic pig 2 compared with wild-type controls.

but also H-D antigen. The H-D antigen, which contains *N*-glycolylneuraminic acid (NeuNGc) is widely distributed in mammalian species, except for humans. The expression of NeuNGc is controlled by CMP-*N*-acetylneuraminic acid (CMP-NeuNAc) hydroxylase activity. The absence of NeuNGc in human glycoconjugates is caused by a partial deletion in the gene that encodes CMP-NeuNAc hydroxylase (42–45). Therefore,

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TABLE II
Heterotopic heart transplantation, from pig to cynomolgus monkey

Donor	Recipient	Anti-PEC titer (IgM)	Anti-PEC titer (IgG)	CH50	Survival time
				units	
Control					
1		83.1	27.8	149.6	25 min
2		38.1	18.8	200.3	2 h 45 min
Transgenic					
1		175.3	71.1	128.4	2 h 33 min
2		33.5	32.4	164.3	>4 h[a]

[a] The graft was beating normally when removed.

this epitope has the potential to become one of the largest epitopes in the pig to human xenotransplantation after α1,3GT is knocked out. Fortunately, it is possible for GnT-III to reduce the levels of the H-D antigen because GnT-III acts not only on α-Gal but any other unknown epitopes as well.

Increasing evidence suggests that NK cells play a critical role in swine to human xenotransplantation (46, 47). Thus, an investigation of the effect of enzymatic remodeling of a glycoantigen, especially α-Gal, to NK-mediated direct cytotoxicity was carried out using the PEC from the transgenic pig. The down-regulation of NK cell-mediated direct killing was also observed in the PEC, consistent with the *in vitro* data reported in our previous study (36).

Some reports revealed that the cynomolgus monkey may not be an ideal substitute for the human recipient in terms of the hyperacute rejection of pig organs. The natural antibody titer to PEC and the classical pathway of the complement, CH50, were therefore assessed and compared with several human volunteers. However, these data, relative to the cynomolgus monkey that we used here, are no lower than those of humans (data not shown). Other reports have indicated that transplanted pig hearts were rejected within 1 h in the case of recipient cynomolgus monkeys. In this study, the control 1 donor heart was rejected at 25 min in the recipient cynomolgus monkey with an average CH50 unit and a slightly high IgM titer. Therefore, the case of control 2, with a relatively low level of IgM and IgG titer (48), which was rejected in 165 min after transplantation, might be a rare case for the pig to cynomolgus monkey combination.

The transplantation experiments do not permit specific conclusions to be drawn because of the limited number of experimental animals used. However, the graft survivals can be inferred from the effect of the down-regulated antigenicity of GnT-III. Approximately half diminished antigenicity of GnT-III prolonged the heart graft survival of the pig in the cynomolgus monkey in the case of transgenic pigs. The first case, transgenic 1 graft, rejected at almost the same time as the control 2. However, the recipient monkey in which the transgenic 1 graft was transplanted showed a slightly lower CH50 unit but had 4.6 times and 3.8 times higher IgM and IgG titers, respectively, than that of control 2. On the other hand, in the case of transgenic 2, despite the fact that the recipient monkey had a IgM titer nearly equal to control 2, the graft heart showed a good contraction during the follow-up time, 4 h, without any sign of rejection. The outcome of pig xenografts in primate models has also been shown to correlate with *in vitro* human serum-mediated cytolysis and other assays performed using pig endothelial cells from the transgenic pig.

A variety of strategies have been pursued to eliminate α-Gal from swine tissue including the knock out of the α1,3GT gene. Among these, the gene transfection of α1,2FT has been reported to result in a drastic suppression of α-Gal. Sandrin *et al.* (18) reported an ~70% reduction in α-Gal expression in a pig kidney fibroblast cell. Moreover, Sharma *et al.* (50) demonstrated that this approach is effective in transfected Chinese hamster ovary cells with α1,3GT and α1,2FT. Both reports also indicated that this approach to reduce the α-Gal was quite effective in the transgenic mouse. In addition, transgenic pigs expressing α1,2FT have already been reported by several groups (49–52). However, as a disadvantage, Sepp *et al.* (53) demonstrated that Lewis^X expression was reduced to background levels, whereas the Lewis^Y neoepitope was induced in the case of α1,2FT-expressing pig cells.

On the other hand, as we have reported previously, α2,3ST and α2,6ST dramatically suppress the antigenicity of pig cells to human natural antibodies better than the α1,2FT (19, 20). In addition, we have demonstrated that the combined transfer of GnT-III and α2,3ST appears to comprise ~80% of the down-regulation in xenoantigenicity (54). However, the transgenic mouse and pig carrying a high level of α2,3ST activity or α2,6ST were difficult to produce, judging from our previous experience (data not shown).

Another study suggests that a combined transgenic approach using α-galactosidase and α1,2FT results in the continuous suppression of the α-Gal of donor tissue (55). However, despite the drastic elimination of the α-Gal in the pig cells with the α-galactosidase gene, the transgenic mouse carrying the α-galactosidase alone showed only a 15–25% reduction in the α-Gal (55). At the present time, none of these approaches except for the knockout completely eliminate the expression of the α-Gal. Galili (56) suggests that even a decrease of 95% in the α-Gal expression may not suffice for prevention the rejection of a xenograft. Further approaches might be required to suppress the xenoantigenicity of pig cells and grafts effectively.

Regarding transgenic pig with a glycosyltransferase, four groups have reported on transgenic pigs with α1,2FT. The first report was from Nagoya's group (49). They actually produced transgenic pigs with α1,2FT but could not maintain the transgenic line long enough for a clear analysis. The report from Nextran (50) only analyzed a tail section of the transgenic pig. The next case was reported by Alexion Pharmaceuticals Inc. (51). They used a H2K^b and a cytomegalovirus promoter and obtained a good expression of the α1,2FT gene in most of the transgenic pig tissues and analyzed the modified cell surface carbohydrates, mainly fibroblasts. A 50% reduction of the α-Gal epitopes of the PEC from the α1,2FT transgenic pigs was also shown, but they did not perform the transplantational experiment. The other report by Cown *et al.* (52) described the combined transgenic pigs with CD55/α1,2FT and CD55/CD59/α1,2FT. Although the α1,2FT expressions were relatively weak and did not significantly reduce the α-Gal epitopes, the transgenic kidney expressing these genes, when transplanted into baboon, survived for as long as 5 days. This report suggests the importance of the combination of the complement regulatory proteins and the glycosyltransferases.

The combination approach within glycosyltransferases, such as α2,3ST and GnT-III, or α-galactosidase and α1,2FT, have already been demonstrated to cause the effective suppression of α-Gal. Therefore, the triple combination of GnT-III, fucosyltransferase or sialyltransferase, and α-galactosidase might be

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the best approach to achieving the down-regulation of xenoantigenicity at the present time. Needless to say, to achieve the best approach for overcoming hyperacute rejection, two or three complement regulatory proteins must be added simultaneously to the combined glycosyltransferases.

Collectively, the advantages of the present approach in reducing xenoantigens are as follows. 1) GnT-III is relatively easy to express in transgenic pig. 2) This approach leads to a remodeling of the total antigenicity; that is, not only the α-Gal but also H-D antigen and other unknown epitopes as well. 3) The combined transgenic approach of GnT-III with another glycosyltransferase, such as α2,3ST or α1,2 FT, is very effective (54). 4) Therefore, after an α1,3GT knockout pig becomes feasible, it would be advisable to add this gene to the knockout pig to reduce xenoantigenicity, in addition to the α-Gal. 5) Finally, the expression of the GnT-III in transgenic pigs clearly modifies the surface glycoantigen in most tissues and cells, especially α-Gal, and confers resistance to human natural immunity.

Acknowledgment—We thank Dr. Milton S. Feather for editing this manuscript.

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